

University of Groningen

Data resources and mining tools for reconstructing gene networks in *Lactococcus lactis*

de Jong, Anne; Kok, Jan; Kuipers, Oscar

Published in:
Japanese Journal of Lactic Acid Bacteria

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Publication date:
2011

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

de Jong, A., Kok, J., & Kuipers, O. (2011). Data resources and mining tools for reconstructing gene networks in *Lactococcus lactis*. *Japanese Journal of Lactic Acid Bacteria*, 22(1), 3-14.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Review

Data resources and mining tools for reconstructing gene regulatory networks in *Lactococcus lactis*

Anne de Jong^{1,2,3)}, Jan Kok^{1,2,3)} and Oscar P. Kuipers^{1,2,3)*}

¹⁾ Department of Molecular Genetics, University of Groningen, Groningen Biomolecular Sciences and Biotechnology Institute, Nijenborgh 7, 9747 AG Groningen, the Netherlands

²⁾ Top Institute Food and Nutrition, Wageningen, the Netherlands

³⁾ The Netherlands Kluyver Centre for Genomics of Industrial Fermentations / NCSB, Delft, the Netherlands.

Abstract

DNA is the blueprint and template for tRNA, rRNA, sRNA and mRNA synthesis in all living organisms. Subsequently, the mRNA is translated to proteins a process in which other RNA types, compounds and proteins play important roles. The regulation of transcription and translation is a delicate equilibrium of thousands of factors interacting within an organism. These factors vary from compound- and protein concentrations, to their activity and stability to physical parameters like temperature and pH. Especially the transcription factors and transcription factor binding sites play a central role in the regulation of gene expression. For *lactococci lactis* several data sets on gene expression and regulation are available in databases or literature. Furthermore, the number of (in-) complete sequenced lactococci genomes is increasing, while the majority of the strains will only be studied *in silico*. This review focuses on the visualization and mining of the complex transcription and translation systems via interactive graphics and network reconstruction tools and the amalgamation of DNA microarray data with biological data that together lead to the reconstruction of gene regulatory networks (GRN). These networks are a valuable source of information and knowledge that can be used for studying *L. lactis* physiology and provide clues for improving industrial strains by either non-GMO methods (strain selection, fermentation condition) or by specific engineering.

Key words: DNA microarray, Gene Regulatory Network, Time series, Transcription factor, Transcription factor binding site, *Lactococcus lactis*

Lactococcus lactis

Since 10.000 years humans consume fermented milk

products as a rich source for proteins, small peptides, amino acids, fatty acids, calcium and other valuable nutrients. The milk sugar lactose is fermented to lactic acid; the consequent acidification prevents food spoilage and is probably the major driving force of the success of fermented milk products. Although nowadays other food preservation methods are applied, the acidification of the product is still the major growth inhibitor of bacteria other than Lactococci. The global world market of milk-related products is estimated

*To whom correspondence should be addressed.

Phone : +31-50-3632092

Fax : +31-50-3632348

E-mail : Anne.de.Jong@rug.nl

: Jan.Kok@rug.nl

: O.P.Kuipers@rug.nl

at € 240 billion (IMIS Euromonitor 2006) explaining the high interest of the dairy industry in research on food process management and improvement. To gain more insight in flavour formation, intensive research is taking place to unraveling genomes, transcriptomes, proteomes and (bio-) chemical pathways. The Holy Grail would be to build a predictive *in silico* model of each bacterial strain involved in milk fermentation. Probably this holistic result will not be obtained soon, not even for one of the organisms, but recent work has already led to models describing various relatively simple processes in the bacterial cell. The basis of this research is the availability of genome sequences of lactic acid bacteria but, although many *L. lactis* genomes have been sequenced, mainly by industrial companies, only a few are available in the public domain (from the strains MG1363, IL1403, SK11 and KF147) at "The Integrated Microbial Genomes (IMG) system" ¹⁾. In this review we will discuss the state of the art bioinformatics tools that facilitate the reconstruction and visualization of gene regulatory networks in *L. lactis*.

Gene prediction

Normally, splicing of mRNA does not occur in prokaryotes, which would suggest that prediction of a protein encoding gene (open reading frame (ORF)) is a simple and straight forward process. Although prediction methods like GeneMarkHMM ²⁾, Glimmer 3 ³⁾, TriTISA ⁴⁾, TICO ⁵⁾, Easygene ⁶⁾, MED 2.0 ⁷⁾ and PRODIGAL ⁸⁾ predict up to 90% of the genes correctly in most prokaryotes, still quite a number of genes are incorrect predicted or missed ⁸⁾. All methods struggle with predicting correct translation start point (AUG, GUG and UUG) and use Shine-Dalgarno Ribosomal Binding Site (RBS) motifs to improve the prediction of the translation initiation site. An important difference between coding and non-coding DNA regions is the GC bias and this phenomenon has been used to improve the ORF prediction. For each organism this bias can be calculated and is used to plot reading frame information for detection of the spurious upstream gene region. Furthermore, modern prediction methods use an ORF length threshold of 90-120 bp, because of the high rate of false positives for

smaller genes. Prediction of a small ORF (smaller than 120-150 bases) is still very complicated. In annotation processes small ORFs are habitually discarded when they do not show homology to known proteins. Of course, in this way, novel small genes will never be discovered. BAGEL2 ⁹⁾, a bacteriocin mining tool, circumvents this issue by taking the genomic context into account and is able to discover small ORFs encoding novel lantibiotics. The main resources for annotated genomes, NCBI, EBI and JGI, offer multiple ORF predictions tools made by Glimmer3 and PRODIGAL.

Operon prediction

An operon is defined as a cluster of genes that are translated from one mRNA, which is thus called a polycistronic messenger, and are under control of the same promoter. To accurately predict operon purely on the basis of genome sequences is still a challenging task. Using gene direction, gene spacing and transcription terminator prediction programs in combination with comparative genomics, up to 95% accuracy of operon prediction can be achieved ¹⁰⁾. Currently, databases provide operons predicted in publically accessible genomes. For the *de novo* annotation or re-annotation of operons, UNIPOP provides an easy, quick and accurate way ^{11, 12)}. DOOR ¹³⁾ (Database of prokaryotic Operons) using the algorithm developed by Dam ¹⁴⁾ which is believed to be the most accurate prediction method as described in an intensive comparison study ¹⁵⁾. MicrobesOnline is a product of the Virtual Institute for Microbial Stress and Survival; it provides operons for 620 genomes while OperonDB contains operons for 550 genomes, ODB ¹⁶⁾ for 203 genomes. RegulonDB ¹⁷⁾ basically provides *E. coli* operons and DBTBS ¹⁸⁾ those for *B. subtilis*. While operons in OperonDB ¹⁹⁾, Microbes Online and ODB ¹⁶⁾ are only predicted, operons defined in RegulonDB and DBTBS are mainly based on laboratory experiments.

Transcriptomics

Bacteria often encounter changes in their environment and will adapt the expression of genes

and activity of proteins to optimize growth, which is of utmost importance to survive in a competitive niche. By sensing the changes in the intracellular and/or extracellular environment, the cell can adjust the transcription of genes and subsequently change its protein content, to cope with the new situation. Transcription of genes (gene expression) is tightly regulated and mostly mediated by sigma factors and other *trans*-acting transcription factors (transcription regulator proteins; TFs). Furthermore, micro RNA's and T-boxes or S-boxes, can be involved in mediating the level of transcription. The TFs can be activated or deactivated when they directly or indirectly "sense" changes in concentration of physical constants (e.g. temperature), ions or other molecules in the cell. Besides these types of TFs, two component signal transduction systems (e.g. by phosphorelay) exist that specifically react on external signals ²⁰⁾. DNA microarray studies show the expression of all genes at a defined state of the cells transcriptome. Different growth rates, stress or other conditions and also the co-expression of genes can be used to build a gene-to-gene interactive network including their deduced proteins and subsequent metabolic compounds. The co-regulation of a collection of genes by the same TF is called a regulon.

Transcription factors

Transcription of DNA to mRNA can be activated or repressed when a TF binds to a specific cis-regulatory DNA element (TFBS) in the promoter region of an operon. The DNA binding region of TFs often consists of a conserved Helix-Turn-Helix secondary structure, a motif that is prevalent in TFs of prokaryotes ²¹⁾. Furthermore, it is common that a co-factor molecule is required to activate a TF e.g. branched-chain amino acids (BCAAs) for CodY of *L. lactis* ²²⁾. Such a co-factor influences the secondary or tertiary structure of the TF and thus its DNA binding capability. In general, the type of regulation (activation or repression) depends on the position of the TFBS relative to the binding site of the RNA polymerase. A special class of TFs is formed by the so-called two component systems, where a dedicated sensory kinase activates the TF by phosphorylation.

Transcription regulators in *Lactococcus lactis*

In the genome of *L. lactis* subsp. *cremoris* MG1363 154 genes are annotated as ncoding TFs. Among these, 32 have been studied in either *L. lactis* MG1363 or in *L. lactis* IL1403 and among these, 7 genes encode TFs that are members of a two component system (Table 1). Another 55 TFs have high homology to regulators in other bacteria with known function (not shown) while 68 are unknown TFs annotated as putative, based on conserved protein motifs involved in DNA-binding.

Regulons

Genes or operons that are under control of the same TF are member of a regulon. Although prediction methods for regulons have been substantially

Table 1. Transcriptional regulators studied in *L. lactis* MG1363 and/or IL1403

Gene	Literature
AhrC	MG1363 ^{57, 58)}
ArgR	MG1363 ^{57, 58)}
BusR	^{59, 60)}
CcpA	MG1363 ⁶¹⁾
CodY	MG1363 ^{22, 62)}
ComX	⁶³⁾
CopR	IL1403 ⁶⁴⁾
CtsR	MG1363 ^{65, 66)}
FhuR	IL1403 ⁶⁷⁾
FlpA	MG1363 ⁶⁸⁾
FlpB	MG1363 ⁶⁸⁾
FruR	⁶⁹⁾
GadR	^{70, 71)}
GntR	MG1363 ⁷²⁾
HdiR	MG1363 ⁷³⁾
HisZ	^{74, 75)}
LlrA	MG1363 ⁷⁶⁾
LlrB	MG1363 ⁷⁶⁾
LlrC	MG1363 ⁷⁶⁾
LlrD	⁷⁷⁾
LlrE	MG1363 ⁷⁶⁾
LlrF	MG1363 ⁷⁶⁾
LlrG	MG1363 ⁷⁶⁾
LmrR	MG1363 ^{78, 79)}
MalR	⁸⁰⁾
PhoU	⁸¹⁾
PurR	⁸²⁾
PyrR	MG1363 ⁸³⁾
RcfB	⁸⁴⁾
SpxA	^{85, 86)}
XylR	⁸⁷⁾
ZitR	⁸⁸⁾

improved, they are still far from perfect. A relatively small number of TFs have been studied in detail in *L. lactis* and the same accounts for other well-studied microorganisms such as *E. coli* and *B. subtilis*. By using comparative genomics, regulons can be predicted in bacterial genomes. Although this is true for many regulons, the procedure can also lead to incorrect regulon calling. Comparative genomics fails for the diverged regulon of the *B. subtilis* TF CodY, which regulates quite different genes than does CodY of *L. lactis*. Despite this drawback several regulon databases are available that are based on comparative genomics of regulons (Table 2). Probably the most extended and accurate databases of regulons are DBTBS and RegulonDB. RegulonDB is the oldest and is specifically developed for *Escherichia coli* K12. It is updated on a regular basis starting from 1998¹⁷⁾. Furthermore, the RegulonDB web server offers the Nebulon regulon mining tool for other but a limited number of prokaryotes. The first release of the database of transcriptional regulation in *B. subtilis* (DBTBS) dates from 1999 and is regularly updated¹⁸⁾. The latest update brought the total number of *B. subtilis* TFs to 120, promoters to 1475 and regulated operons to 736. In the meantime from which 463 of these operons have been experimentally validated. The most comprehensive manually curated regulon database of *L. lactis* is MolgenRegDB, which is available via the PePPER webserver (<http://pepper.molgenrug.nl>). Together, RegulonDB, DBTBS and MolgenRegDB are currently the major resources for regulon network mining in dedicated to prokaryotes. PRODORIC²³⁾ is the most extended generic and manually curated database on gene regulation in prokaryotes in general. Besides regulon information it includes TFBSs and bioinformatics tools for prediction, analysis and visualization of gene regulatory networks

Table 2. Main webserver for regulon databases and mining

Web-server	Organisms	Main Tools	url
DBTBS	<i>Bacillus subtilis</i> 168	-	http://dbtbs.hgc.jp/
RegulonDB	<i>Escherichia coli</i> K12	Nebulon	http://regulondb.ccg.unam.mx/
Regprecise	+/- 50	-	http://regprecise.lbl.gov/
PRODORIC	>200	Virtual Footprint	http://prodoric.tu-bs.de/
FITBAR	>500	PSSM/LLM	http://archaea.u-psud.fr/fitbar/
ProdoNet	>500	Network	http://www.prodonet.tu-bs.de
RegAnalyst	Mycos	MoPP	http://www.nii.ac.in/~deepak/RegAnalyst/
PePPER	All NCBI	Genomics	http://pepper.molgenrug.nl/

using ProdoNet²⁴⁾. The recently launched web server RegPrecise²⁵⁾ gives access to a database containing a collection of manually curated regulons grouped together by similar properties such as TFs, biological process and metabolic pathway. The database is limited to six taxonomic groups of closely related genomes (*Shewanella*, *Staphylococcus*, *Streptococcus*, Thermotogales, Bacillales and Desulfovibrionales).

Regulons in *Lactococcus lactis*

Each of the 154 TFs of *L. lactis* MG1363 will probably regulate the transcription of one or more genes. As mentioned before, the functionality of 32 TFs of *L. lactis* strain MG1363 or *L. lactis* IL1403 has been reported in literature, using techniques ranging from DNA microarrays to DNA footprinting. Although the two lactococcal strains are closely related, not all their regulators and regulons are present or similar. Comparative genomic studies showed that large regulons (those of CodY, CcpA, CmbR, CesSR, ArgR, and PurR) as well as small regulons (those of RcfB, ZirR, BusR, LmrR) are well conserved in the two strains. Likewise, the majority of the TFs are highly conserved between MG1363 and IL1403. From the total of 154 TFs in *L. lactis* MG1363, 22 are not present in *L. lactis* IL1403 while 20 out of the 143 TFs identified in *L. lactis* IL1403 are not found in MG1363 (Table 3). Furthermore, some regulators, like CmhR, appear to be less conserved (55%) between the two strains. Furthermore, it has been shown (unpublished results) that the CmhR regulons of both strains overlap but are not identical. The conservation of regulons between closely related strains is illustrated by the CmbR regulon of cysteine and methionine biosynthesis which has been studied in detail in *L. lactis* IL1403²⁶⁾ and in *L. lactis* MG1363²⁷⁾. Comparative genomics of

both CmbR regulons showed that 16 out of 17 proteins in the IL1403 CmbR regulon have high homology to MG1363 proteins, suggesting the good conservation of the regulon between these two species (see Table 4).

Transcription Factor Binding Sites (TFBSs)

The relation of conserved TFBSs in promoter regions with the expression of genes was already

Table 3. Comparative genomics of TF in IL1403 and MG1363

MG1363 TFs not in IL1403	IL1403 TFs not in MG1363
ArsR	CitR
Limg_0069	LlrH
Limg_0089	Pi103
Limg_0147	Pi104
Limg_0161	Pi356
Limg_0424	Pi357
Limg_0432	Ps111
Limg_0709	Ps314
Limg_0865	RliB
Limg_0962	RliDA
Limg_1051	RliDB
Limg_1324	RlrA
Limg_1527	RlrC
Limg_1868	RmaF
Limg_1903	RmaJ
Limg_2334	YcfA
Limg_2441	YdcG
Ps116	YneE
Ps205	YrfA
Ps404	YwjD
Ps408	
RgrB	

discovered in 1983²⁸⁾. Experimental methods like Electrophoretic Mobility Shift Assays (EMSA), Surface Plasmon Resonance (SPR), nuclease protection assays (footprinting) and Chromatine Immuno Precipitation (ChIP) can all be used to prove that an interaction exists between TF and DNA. Experimentally validated TFBSs have been described in literature and are available via publicly accessible databases (DBTBS, RegulonDB, PRODORIC, RegPrecise, PePPER). Besides experiments on protein-DNA interaction, TFBS discovery algorithms have been developed to predict conserved DNA regions. Motif mining is based on a collection of genes having a correlation; subsequently a search starts for the uncovering of conserved DNA patterns in the upstream intergenic region of the gene or the operon to which the gene belongs. The gene-to-gene correlation can be derived from transcriptome data, regulon data or from functional relations like metabolic pathways, COGs or GO classes. An overview of the major prediction algorithms is shown in Table 5.

TFBS mining is a complex and challenging task for biologists and bioinformaticians, but many different approaches in developing TFBS discovery tools are being undertaken and progress is very encouraging.

Regulon prediction in prokaryotes

The number of fully sequenced bacterial genomes is rapidly increasing thanks to Next Generation Sequencing (NGS) technology, but experimental

Table 4. Comparative genomics of the CmbR regulon in *L. lactis* strains IL1403 and MG1363

IL1403 gene	MG1363 gene	IL1403 locus	MG1363 locus	Description
<i>cysD</i>	<i>cysD</i>	LL0073	llmg_0091	O-acetylhomoserine sulphydrylase
<i>cysK</i>	<i>cysK</i>	LL0782	llmg_1775	Cysteine synthase
<i>cysM</i>	<i>llmg_0508</i>	LL0540	llmg_0508	Cysteine synthase
<i>metA</i>	<i>metA</i>	LL1918	llmg_2182	Homoserine O-succinyltransferase
<i>metB1</i>	<i>metB1</i>	LL1917	llmg_2181	Cystathionine gamma-synthase
<i>metB2</i>	<i>metC</i>	LL0181	llmg_1776	Prenyl transferase
<i>plpA</i>	<i>plpA</i>	LL0318	llmg_0335	Outer membrane lipoprotein
<i>plpB</i>	<i>plpB</i>	LL0319	llmg_0336	Outer membrane lipoprotein
<i>plpC</i>	<i>plpC</i>	LL0320	llmg_0338	Outer membrane lipoprotein
<i>plpD</i>	<i>plpD</i>	LL0321	llmg_0340	Outer membrane lipoprotein
<i>ydCB</i>	<i>metN</i>	L121289	llmg_0341	amino acid ABC transporter ATP binding protein
<i>ydCD</i>	<i>llmg_0343</i>	LL0324	llmg_0343	UPF0397 protein ydcD
<i>yhcE</i>	<i>metE2</i>	LL0718	llmg_1849	Putative uncharacterized protein yhcE
<i>yjgC</i>	<i>llmg_1593</i>	LL0937	llmg_1593	Amino acid ABC transporter substrate binding
<i>yjgD</i>	<i>llmg_1591</i>	LL0938	llmg_1591	Amino acid ABC transporter permease protein
<i>yjgE</i>	<i>llmg_1590</i>	LL0939	llmg_1590	Amino acid ABC transporter ATP binding protein
<i>ytjE</i>	-	LL1916		Aminotransferase

Table 5. TFBS prediction and mining methods

Method	url
MEME	http://meme.sdsc.edu/
GLAM2	http://meme.sdsc.edu/
MDScan	http://robotics.stanford.edu/~xslu/MDscan/
Genome2D	http://genome2D.molgenrug.nl
DISCLOSE	http://bioinformatics.biol.rug.nl/standalone/disclose/
SCOPE	http://genie.dartmouth.edu/scope/
MotifMiner	http://mnm.engr.uconn.edu/
YMF	http://wingless.cs.washington.edu/YMF/
PhyloGibbs	http://www.phylogibbs.unibas.ch
Clover	http://zlab.bu.edu/clover/
TAMO	http://fraenkel.mit.edu/TAMO/
SwissRegulon	http://www.swissregulon.unibas.ch
MARA	http://www.swissregulon.unibas.ch
TCS	http://www.swissregulon.unibas.ch
RSAT	http://rsat.ulb.ac.be/rsat/index.html

verification of the loads of annotations and in particular the regulons is still a huge task. The *in silico* prediction of regulons is usually based on operons that share the same TFBS, information is supplemented with comparative genomics to known regulons. Powerful tools have been developed for prokaryotes; these combine regulon knowledge databases with modern TFBS search algorithms. As mentioned before PRODORIC contains an extended database of regulon data of many organisms and, in addition, offers the tool "virtual footprint" to mine for novel regulons. Whereas FITBAR²⁹⁾ is more dedicated to TFBS mining and discovery, RegAnalyst³⁰⁾ and ProdoNet²⁴⁾ are webserver enabling integration of data on proteomics and metabolic pathways and provide subsequent graphical representation of networks. An overview of webserver dedicated to regulons and TFBS is shown in Table 2.

Techniques for measuring and discovering *cis*-regulatory elements

The combined DNA microarray and ChIP-on-Chip data is the major global source for reconstruction of gene regulation networks (GRNs). Detailed information can be retrieved from EMSA and SPR studies on TF-DNA interactions. In addition, footprinting is used to locate the exact binding site of the TF on its target DNA. Non-protein regulatory elements like microRNA (miRNA) and small interfering RNA (siRNA) have

recently been discovered as being very important in gene regulation in prokaryotes and they can be discovered by deep RNA sequencing. Novel promising techniques are being developed such as, *in vivo* digital genomic footprinting: by mapping DNaseI cleavage sites using massive parallel DNA sequencing, the locations of *cis*-regulatory elements on the genome of *Saccharomyces cerevisiae* were uncovered³¹⁾. Novel bioinformatics tools and genetic techniques have led to a valuable resource for GRN reconstruction and subsequently to get insight in the complex regulation machinery.

Visualization

In contrast to computers, humans are not able handling large quantities of numbers but are evolved more towards recognition of patterns, colors and movements. Therefore, extensive datasets have to be transformed into a format that is more comprehensible for human. Furthermore, integration of data originating from different sources should be integrated in such a way that it enables understanding the interactions and the relations within these sources. The TIGR4 Multi Experiment Viewer (TM4) is one of the best freeware tools to analyze and visualize DNA microarray data. For integration of such data in metabolic pathways, the KEGG webserver offers several tools; one can either use the website directly or link information of KEGG to stand-alone tools (DISCLOSE, Genome2D, TM4, Cytoscape). The software package Genome2D³²⁾ combines data derived from DNA microarray studies, motif mining, prediction of operons, terminator and DNA motifs to genome annotation data and creates a graphical representation of the genome in concert with the various resources. For studying complex relations between multiple data sources, the Cytoscape platform³³⁾ enables construction of GRNs and sophisticated data mining.

Time-resolved (chrono) transcriptomics

Bacteria are equipped with an elaborate sensory system that can detect most stressful conditions. They use the obtained information to redirect their metabolism to meet the challenges. Adaptation to

changes in their environment can be detected at the level of the transcriptome through changes in gene expression. To get an insight in the responses to (environmental) changes taking place during batch fermentation and to reveal classes of genes (genes as part of pathways, operons, regulons, COGs) that are important during bacterial growth, transcriptome studies were performed on *L. lactis* during growth in rich media (GM17 and milk) (unpublished results). This transcriptome time-series analysis provided insights in gene expressions over time and showed that *L. lactis* adapted quickly to environmental changes. As an example: the expression profiles during growth in milk of the genes encoding for the oligopeptide permease system (*opp*) and its regulator gene *codY* encoding for the TF CodY is shown in Figure 1. The transcription profiles show that expression of *opp* is stimulated during late exponential growth in milk. The increase in expression of *codY* precedes that of *opp*, which points to a specific response time of the regulon or to the availability of co-factors (branched-chain amino acids) needed to establish an active CodY regulator. A low correlation between the TF and its regulon in chrono transcriptomics is not unique for *codY*, it is more common and does not allow to automatically link TFs to the network when network reconstruction is done purely on the basis of DNA microarray data¹⁸⁾.

Data resources for reconstructing Gene Regulatory Networks

Building a GRN relies on data describing interactions or associations between elements. The two resources for a GRN are derived from; i) gene-to-gene interactions based on DNA microarray data, and ii) databases containing biological data as listed in Table 6.

Gene Regulatory Network Reconstruction

Inferring GRN from DNA microarray data is a challenging task and many papers handling this problem have been published in recent years³⁴⁾. The basic idea of these networks is to gain insight into interactions between biological processes. The number of computational methods that reconstruct such GRNs (synonym; Transcription Regulatory Networks (TRNs)) from genome-wide expression data is rapidly increasing but the methods are not always an improvement³⁵⁾. In principle two main approaches for reconstruction networks are being used, namely Bayesian networks or Graphical Gaussian networks. This last model, first used in GeneNet³⁶⁾, is currently considered as the most reliable approach and is used

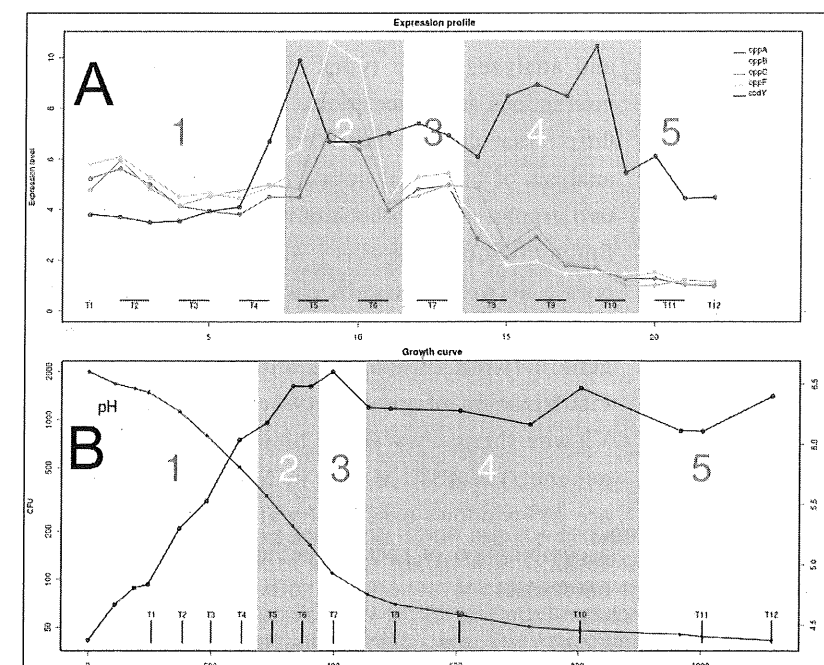


Figure 1. Expression profile of the *opp* operon of *L. lactis* MG1363. Results derived from a time-series experiment of *L. lactis* MG1363 grown for 1100 min at 30 °C in milk. Samples were taken for DNA microarray analysis, as indicated by horizontal or vertical lines on the x-axis numbered from T1 to T12. Grey blocks in the graphs indicated the growth phases: 1. early exponential; 2. late exponential; 3. transition; 4. early stationary; 5. late stationary. A) Expression profiles of *oppABCF* and *codY*. B) Growth and acidification of MG1363.

Table 6. Main resources for genome annotation

Name	Class	url
NCBI	Genome resource	http://www.ncbi.nlm.nih.gov/
EBI	Genome Review	http://www.ebi.ac.uk/
EMBL	Genome resource	http://www.ebi.ac.uk/embl/
JGI	Integrated Microbial Genomes	http://img.jgi.doe.gov/
COG	Classification	http://www.ncbi.nlm.nih.gov/COG/
GO	Classification	http://www.geneontology.org/
KEGG	Metabolic pathways	http://www.genome.jp/kegg/
PSI	Structural Biology of proteins	http://www.sbk.org/
PFAM	Protein	http://pfam.sanger.ac.uk/
STRING	Protein protein interaction	http://string-db.org/
Uniprot	Protein functional information	http://www.uniprot.org/
Microbes online	Genome functional annotation	http://www.microbesonline.org/

as the fundament in current network reconstruction methods. The following section describes the state of GRN building systems to date.

Gene Regulation Network inference methods

ARACNE is an algorithm for the reconstruction of GRNs in a mammalian cellular context³⁷⁾ subsequently this package was further improved (TimeDelay-ARACNE) to use time-series data as a source information to reconstruct gene networks³⁸⁾. Whereas ARACNE is dedicated to mammals, SEREND (SEmi-supervised REgulatory Network Discoverer)³⁹⁾, SIRENE⁴⁰⁾, and DISTILLER⁴¹⁾ are gene network reconstruction systems developed and tested specific for data derived from experiments with *E. coli*. Subsequent experimental validation of the performance of these methods at the genome scale had to be done. Therefore, an extensive validation study comparing the transcriptional regulation database RegulonDB with 445 Affymetrix DNA microarrays of the transcriptional regulation of *E. coli* was done using CLR⁴²⁾. The predicted interactions for three TFs was tested with chromatin immune precipitation (ChIP); 21 novel interactions derived from their analysis using CLR were confirmed. CLR and ARACNE were further improved in MRNET⁴³⁾. Afterwards C3NET claims that it outperforms MRNET by inferring the conservative causal core

of gene regulatory networks⁴⁴⁾. More detailed information of the performance and pro's and cons of these tools have recently be published in a review⁴⁵⁾. Inferring the GRN using a combination of methods as described above is possible using SEBINI, a software environment allowing to evaluate, compare, refine or combine inference techniques^{46, 47)}. Also MINET, like the methods mentioned above, uses transcriptomics data to quantify the statistical evidence of specific gene-to-gene interactions and provides a set of functions to infer mutual information networks from other datasets. The strength of the MINET package⁴⁸⁾ is that it is developed in the statistical environment R and is freely available at the Bioconductor project⁴⁹⁾, which makes it accessible and adaptable for wide-ranging research. Furthermore, MINET claims not to outperform other packages but uses mutual information from four different entropy estimators (empirical, Miller-Madow, Schurmann-Grassberger and shrink) and four inference methods (ARACNE, CLR and MRNET).

Visualization of networks

Mainly three methods are used to visualize networks: i) via web-servers; ii) via stand alone package; iii) using algorithms in a visualization platform (e.g., Cell Designer Cytoscape). The webbased system VisANT^{50, 51)} is one of the few visualization tools for biological networks. VisANT visualizes and analyzes many types of networks of biological interactions and associations. The NeAT webserver⁵²⁾ (<http://rsat.ulb.ac.be/neat/>) provides a toolbox for the analysis of biological networks (comparison between two graphs, neighborhood of a set of input nodes, path finding, cliques discovery and mapping of clusters onto a network), clusters, classes and pathways. The well known GeneNet system uses a database on gene network components and provides automated visualization of gene networks via the GeneNet Viewer (<http://www.mgs.bionet.nsc.ru/mgs/gnw/genenet/>). CAST, MAST in MTOM and SEED⁵³⁾ are MS-windows-based software packages and offer a combination of reconstruction and visualization of networks.

Network reconstruction and visualization

A GRN describes the interactions between TFs and the genes they regulate⁵⁴⁾. Time-series and perturbation transcriptome data derived from DNA microarray studies provide knowledge of timing of gene expression, as well as gene-to-gene and gene-to-class correlation. Inference of GRNs based on DNA microarray studies is referred to as reverse engineering, as the obtained gene expression levels are the outcome of gene regulation. Unfortunately, the ultimate network inference method still does not exist³⁵⁾ meaning that a good choice of statistics should be made, one that reflects the co-expression of genes and gene classes for the given experiments. Furthermore, due to the fact that expression of TF genes does not always coincide with that of the TFs regulate, regulon network reconstruction will often be limited to TF targets. Besides this, reliable TF studies other than DNA microarray studies are necessary to complement the GRN.

Here illustrate the GRN reconstruction using our knowledge of *L. lactis* in combination with chrono transcriptomics data. The regulons of *L. lactis* are shown in a Cytoscape network visualization (Fig. 2) using the most comprehensive database on *L. lactis*: MolgenRegDB (ref PePPER; de Jong, in preparation). This relatively simple network shows how the

regulons of the TFs CodY, CcpA, ArgR and AhrC are interconnected. To be able to visualize co-expression of genes, a Pearson correlation was calculated between the genes using time-series transcriptional data. Genes having a Pearson correlation *p*-value higher than or equal to 0.80 are considered as co-expressed. The members of the CmbR regulon are shown in Figure 6B, where the width of the lines represents the degree of co-expression (*p*-value). The study of CmbR, which regulates methionine and cysteine metabolism, was done in *L. lactis* IL1403⁵⁵⁾, while the genes of *L. lactis* MG1363 were derived from comparative genomics using PePPER. On the basis of highly interconnected Pearson correlated nodes, four sub-graphs (cliques) of the CmbR regulon emerged: i) *metC*, *cysK*; ii) *plpABCD*, *dar*; iii) *lmg_1589-1591*, *lmg_2294-2295*, *lmg_0352*, *metB1* and iv) a group that does not show expression correlation at all. This last group of genes was found to be related to CmbR on the basis of DNA microarray studies in *L. lactis* IL1403 and this relation could not be re-established using the MG1363 chrono transcriptomics data. The example mentioned above shows the power of GRN reconstruction, while even more data can still be linked to the network. A logical subsequent step is to link metabolic processes to the network. The data source can either be complete metabolic processes derived from KEGG pathways or metabolic reactions derived from the reaction

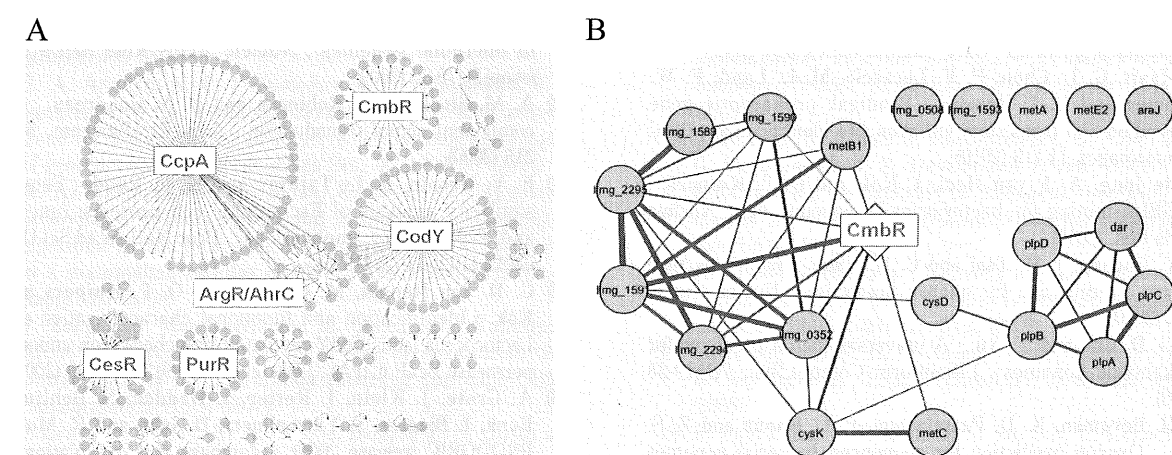


Figure 2. *L. lactis* regulons and CmbR regulon cliques
Cytoscape Network of known regulons in *L. lactis* MG1363. A) The known regulons in *L. lactis*; diamond shapes are TFs and circle shapes are genes. An arrow indicates activation and a blunt-end line shows repression of the gene by the TF. B) All CmbR regulon members connected via Pearson correlations derived from time-series transcriptomics data. The thickness of the lines (edges) corresponds to the strength of co-expression.

database. For *L. lactis* MG1363 a complete model was built by coupling the production and consumption of compounds by enzymes to the reaction database⁵⁶⁾. This production and/or consumption of compounds can directly be integrated into the Cytoscape model by linking enzyme functions to genes.

Conclusion

The current state of bio-informatics algorithms

References

- 1) V. M. Markowitz, I. M. Chen, K. Palaniappan, K. Chu, E. Szeto, Y. Grechkin, A. Ratner, I. Anderson, A. Lykidis, K. Mavromatis, N. N. Ivanova and N. C. Kyrpides. : The integrated microbial genomes system: an expanding comparative analysis resource , Nucleic Acids Res., 38(Database issue),D382-90 (2010)
- 2) R. K. Azad and M. Borodovsky. : Probabilistic methods of identifying genes in prokaryotic genomes: connections to the HMM theory , Brief Bioinform, 5(2),118-130 (2004)
- 3) A. L. Delcher, K. A. Bratke, E. C. Powers and S. L. Salzberg. : Identifying bacterial genes and endosymbiont DNA with Glimmer , Bioinformatics, 23(6),673-679 (2007)
- 4) G. Q. Hu, X. Zheng, H. Q. Zhu and Z. S. She. : Prediction of translation initiation site for microbial genomes with TriTISA , Bioinformatics, 25(1),123-125 (2009)
- 5) M. Tech, B. Morgenstern and P. Meinicke. : TICO: a tool for postprocessing the predictions of prokaryotic translation initiation sites , Nucleic Acids Res., 34(Web Server issue),W588-90 (2006)
- 6) T. S. Larsen and A. Krogh. : EasyGene—a prokaryotic gene finder that ranks ORFs by statistical significance , BMC Bioinformatics, 4, 21 (2003)
- 7) H. Zhu, G. Q. Hu, Y. F. Yang, J. Wang and Z. S. She. : MED: a new non-supervised gene prediction algorithm for bacterial and archaeal genomes , BMC Bioinformatics, 8,97 (2007)
- 8) D. Hyatt, G. L. Chen, P. F. Locascio, M. L. Land, F. W. Larimer and L. J. Hauser. : Prodigal: prokaryotic gene recognition and translation initiation site identification , BMC Bioinformatics, 11,119 (2010)
- 9) A. de Jong, A. J. van Heel, J. Kok and O. P. Kuipers. : BAGEL2: mining for bacteriocins in genomic data, Nucleic Acids Res., (2010)
- 10) L. Y. Chuang, J. H. Tsai and C. H. Yang. : Binary particle swarm optimization for operon prediction , Nucleic Acids Res., (2010)
- 11) G. Li, D. Che and Y. Xu. : A universal operon predictor for prokaryotic genomes , J.Bioinform Comput.Biol., 7(1),19-38 (2009)
- 12) N. H. Bergman, K. D. Passalacqua, P. C. Hanna and Z. S. Qin. : Operon prediction for sequenced bacterial genomes without experimental information , Appl.Environ.Microbiol., 73(3),846-854 (2007)
- 13) F. Mao, P. Dam, J. Chou, V. Olman and Y. Xu. : DOOR: a database for prokaryotic operons , Nucleic Acids Res., 37(Database issue),D459-63 (2009)
- 14) P. Dam, V. Olman, K. Harris, Z. Su and Y. Xu. : Operon prediction using both genome-specific and general genomic information , Nucleic Acids Res., 35(1),288-298 (2007)
- 15) R. W. Brouwer, O. P. Kuipers and S. A. van Hijum. : The relative value of operon predictions , Brief Bioinform, 9(5),367-375 (2008)
- 16) S. Okuda and A. C. Yoshizawa. : ODB: a database for operon organizations, 2011 update , Nucleic Acids Res., (2010)
- 17) S. Gama-Castro, H. Salgado, M. Peralta-Gil, A. Santos-Zavaleta, L. Muniz-Rascado, H. Solano-Lira, V. Jimenez-Jacinto, V. Weiss, J. S. Garcia-Sotelo, A. Lopez-Fuentes, L. Porron-Sotelo, S. Alquicira-Hernandez, A. Medina-Rivera, I. Martinez-Flores, K. Alquicira-Hernandez, R. Martinez-Adame, C. Bonavides-Martinez, J. Miranda-Rios, A. M. Huerta, A. Mendoza-Vargas, L. Collado-Torres, B. Taboada, L. Vega-Alvarado, M. Olvera, L. Olvera, R. Grande, E. Morett and J. Collado-Vides. : RegulonDB version 7.0: transcriptional regulation of Escherichia coli K-12 integrated within genetic sensory response units (Sensor Units) , Nucleic Acids Res., (2010)
- 18) N. Sierro, Y. Makita, M. de Hoon and K. Nakai. : DBTBS: a database of transcriptional regulation in Bacillus subtilis containing upstream intergenic conservation information , Nucleic Acids Res., 36(Database issue),D93-6 (2008)
- 19) M. Pertea, K. Ayanbule, M. Smedinghoff and S. L. Salzberg. : OperonDB: a comprehensive database of predicted operons in microbial genomes , Nucleic Acids Res., 37(Database issue),D479-82 (2009)
- 20) A. M. Stock, V. L. Robinson and P. N. Goudreau. : Two-component signal transduction , Annu.Rev.Biochem., 69,183-215 (2000)
- 21) E. V. Koonin, R. L. Tatusov and K. E. Rudd. : Sequence similarity analysis of Escherichia coli proteins: functional and evolutionary implications , Proc.Natl.Acad.Sci.U.S.A., 92(25),11921-11925 (1995)
- 22) C. D. den Hengst, M. Groeneveld, O. P. Kuipers and J. Kok. : Identification and functional characterization of the Lactococcus lactis CodY-regulated branched-chain amino acid permease BcaP (CtrA) , J.Bacteriol., 188(9),3280-3289 (2006)
- 23) A. Grote, J. Klein, I. Retter, I. Haddad, S. Behling, B. Bunk, I. Biegler, S. Yarmolinetz, D. Jahn and R. Munch. : PRODORIC (release 2009): a database and tool platform for the analysis of gene regulation in prokaryotes , Nucleic Acids Res., 37(Database issue),D61-5 (2009)
- 24) J. Klein, S. Leupold, R. Munch, C. Pommerenke, T. Johl, U. Karst, L. Jansch, D. Jahn and I. Retter. : ProdoNet: identification and visualization of prokaryotic gene regulatory and metabolic networks , Nucleic Acids Res., 36(Web Server issue),W460-4 (2008)
- 25) P. S. Novichkov, O. N. Laikova, E. S. Novichkova, M. S. Gelfand, A. P. Arkin, I. Dubchak and D. A. Rodionov. : RegPrecise: a database of curated genomic inferences of transcriptional regulatory interactions in prokaryotes , Nucleic Acids Res., 38(Database issue),D111-8 (2010)
- 26) B. Sperandio, P. Polard, D. S. Ehrlich, P. Renault and E. Guedon. : Sulfur amino acid metabolism and its control in Lactococcus lactis IL1403, J.Bacteriol., 187(11),3762 (2005)
- 27) M. Fernandez, M. Kleerebezem, O. P. Kuipers, R. J. Siezen and R. van Kranenburg. : Regulation of the metC-cysK Operon, Involved in Sulfur Metabolism in Lactococcus lactis , J.Bacteriol., 184(1),82 <last_page> 90 (2002)
- 28) R. G. Clerc, P. Bucher, K. Strub and M. L. Birnstiel. : Transcription of a cloned Xenopus laevis H4 histone gene in the homologous frog oocyte system depends on an evolutionary conserved sequence motif in the -50 region , Nucleic Acids Res., 11(24),8641-8657 (1983)
- 29) Jacques Oberto. : FITBAR: a web tool for the robust prediction of prokaryotic regulons, BMC Bioinformatics, 11(1),554 (2010)
- 30) D. Sharma, D. Mohanty and A. Surolia. : RegAnalyst: a web interface for the analysis of regulatory motifs, networks and pathways , Nucleic Acids Res., 37(Web Server issue),W193-201 (2009)
- 31) J. R. Hesselberth, X. Chen, Z. Zhang, P. J. Sabo, R. Sandstrom, A. P. Reynolds, R. E. Thurman, S. Neph, M. S. Kuehn, W. S. Noble, S. Fields and J. A. Stamatoiyannopoulos. : Global mapping of protein-DNA interactions in vivo by digital genomic footprinting , Nat.Methods, 6(4),283-289 (2009)
- 32) R. Baerends, W. Smits, A. De Jong, L. Hamoen, J. Kok and O. Kuipers. : Genome2D: a visualization tool for the rapid analysis of bacterial transcriptome data, Genome Biol., 5(5),R37 (2004)
- 33) M. Kohl, S. Wiese and B. Warscheid. : Cytoscape: software for visualization and analysis of biological networks , Methods Mol.Biol., 696,291-303 (2011)
- 34) F. Jaffrezic and G. Tosser-Klopp. : Gene network reconstruction from microarray data , BMC Proc., 3 Suppl 4,S12 (2009)
- 35) R. De Smet and K. Marchal. : Advantages and limitations of current network inference methods , Nat.Rev.Microbiol., 8(10),717-729 (2010)
- 36) E. A. Ananko, N. L. Podkolodny, I. L. Stepanenko, E. V. Ignatieva, O. A. Podkolodnaya and N. A. Kolchanov. : GeneNet: a database on structure and functional organisation of gene networks , Nucleic Acids Res., 30(1),398-401 (2002)
- 37) A. A. Margolin, I. Nemenman, K. Basso, C. Wiggins, G. Stolovitzky, R. Dalla Favera and A. Califano. : ARACNE: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context , BMC Bioinformatics, 7 Suppl 1,S7 (2006)
- 38) P. Zoppoli, S. Morganella and M. Ceccarelli. : TimeDelay-ARACNE: Reverse engineering of gene networks from time-course data by an information theoretic approach , BMC Bioinformatics, 11,154 (2010)
- 39) J. Ernst, Q. K. Beg, K. A. Kay, G. Balazsi, Z. N. Oltvai and Z. Bar-Joseph. : A semi-supervised method for predicting transcription factor-gene interactions in Escherichia coli , PLoS Comput.Biol., 4(3),e1000044 (2008)
- 40) F. Mordelet and J. P. Vert. : SIRENE: supervised inference of regulatory networks , Bioinformatics, 24(16),i76-82 (2008)
- 41) K. Lemmens, T. De Bie, T. Dhollander, S. C. De Keersmaecker, I. M. Thijs, G. Schoofs, A. De Weerd, B. De Moor, J. Vanderleyden, J. Collado-Vides, K. Engelen and K. Marchal. : DISTILLER: a data integration framework to reveal condition dependency of complex regulons in Escherichia coli , Genome Biol., 10(3),R27 (2009)
- 42) J. J. Faith, B. Hayete, J. T. Thaden, I. Mogno, J. Wierzbowski, G. Cottarel, S. Kasif, J. J. Collins and T. S. Gardner. : Large-scale mapping and validation of Escherichia coli transcriptional regulation from a compendium of expression profiles , PLoS Biol., 5(1),e8 (2007)
- 43) P. E. Meyer, K. Kontos, F. Lafitte and G. Bontempi. : Information-theoretic inference of large transcriptional regulatory networks , EURASIP J.Bioinform Syst.Biol., 7,9879 (2007)
- 44) G. Altay and F. Emmert-Streib. : Inferring the conservative causal core of gene regulatory networks , BMC Syst.Biol., 4,132 (2010)
- 45) T. M. Venancio and L. Aravind. : Reconstructing prokaryotic transcriptional regulatory networks: lessons from actinobacteria , J.Biol., 8(3),29 (2009)
- 46) R. Taylor and M. Singhal. : Biological Network Inference and analysis using SEBINI and CABIN , Methods Mol.Biol., 541,551-576 (2009)
- 47) R. C. Taylor, A. Shah, C. Treatman and M. Blevins. : SEBINI: Software Environment for Biological Network Inference , Bioinformatics, 22(21),2706-2708 (2006)
- 48) P. E. Meyer, F. Lafitte and G. Bontempi. : minet: A R/Bioconductor package for inferring large transcriptional networks using mutual information , BMC Bioinformatics, 9,461 (2008)
- 49) R. C. Gentleman, V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang and J. Zhang. : Bioconductor: open software development for computational biology and bioinformatics , Genome Biol., 5(10),R80 (2004)
- 50) Z. Hu, J. Mellor, J. Wu, T. Yamada, D. Holloway and C. Delisi. : VisANT: data-integrating visual framework for biological networks and modules , Nucleic Acids Res., 33(Web Server issue),W352-7 (2005)
- 51) Z. Hu, J. H. Hung, Y. Wang, Y. C. Chang, C. L. Huang, M. Huyck and C. DeLisi. : VisANT 3.5: multi-scale network visualization, analysis and inference based on the gene ontology , Nucleic Acids Res., 37(Web Server issue),W115-21 (2009)
- 52) S. Brohee, K. Faust, G. Lima-Mendez, O. Sand, R. Janky, G. Vanderstocken, Y. Deville and J. van Helden. : NeAT: a toolbox for the analysis of biological networks, clusters, classes and pathways , Nucleic Acids Res., 36(Web Server issue),W444-51 (2008)
- 53) A. Li and S. Horvath. : Network module detection: Affinity search technique with the multi-node topological overlap measure , BMC Res.Notes, 2,142 (2009)
- 54) M. Levine and E. H. Davidson. : Gene regulatory networks for development , Proc.Natl.Acad.Sci.U.S.A., 102(14),4936-4942 (2005)
- 55) B. Sperandio, P. Polard, D. S. Ehrlich, P. Renault and E. Guedon. : Sulfur amino acid metabolism and its control in Lactococcus lactis IL1403, J.Bacteriol., 187(11),3762-3778 (2005)
- 56) R. A. Notebaart, F. H. van Enckevort, C. Francke, R. J. Siezen and B. Teusink. : Accelerating the reconstruction of genome-scale metabolic networks , BMC Bioinformatics, 7,296 (2006)
- 57) R. Larsen, G. Buist, O. P. Kuipers and J. Kok. : ArgR and

- AhrC are both required for regulation of arginine metabolism in *Lactococcus lactis*, *J.Bacteriol.*, 186(4),1147-1157 (2004)
- 58) R. Larsen, J. Kok and O. P. Kuipers. : Interaction between ArgR and AhrC controls regulation of arginine metabolism in *Lactococcus lactis*, *J.Biol.Chem.*, 280(19),19319-19330 (2005)
 - 59) Y. Romeo, J. Bouvier and C. Gutierrez. : Osmotic regulation of transcription in *Lactococcus lactis*: ionic strength-dependent binding of the BusR repressor to the busA promoter, *FEBS Lett.*, 581(18),3387-3390 (2007)
 - 60) Y. Romeo, D. Obis, J. Bouvier, A. Guillot, A. Fourcans, I. Bouvier, C. Gutierrez and M. Y. Mistou. : Osmoregulation in *Lactococcus lactis*: BusR, a transcriptional repressor of the glycine betaine uptake system BusA, *Mol.Microbiol.*, 47(4),1135-1147 (2003)
 - 61) A. L. Zomer, G. Buist, R. Larsen, J. Kok and O. P. Kuipers. : Time-resolved determination of the CcpA regulon of *Lactococcus lactis* subsp. *cremoris* MG1363, *J.Bacteriol.*, 189(4),1366-1381 (2007)
 - 62) C. D. den Hengst, P. Curley, R. Larsen, G. Buist, A. Nauta, D. van Sinderen, O. P. Kuipers and J. Kok. : Probing direct interactions between CodY and the oppD promoter of *Lactococcus lactis*, *J.Bacteriol.*, 187(2),512-521 (2005)
 - 63) S. Wydau, R. Dervyn, J. Anba, S. Dusko Ehrlich and E. Maguin. : Conservation of key elements of natural competence in *Lactococcus lactis* ssp, *FEMS Microbiol.Lett.*, 257(1),32-42 (2006)
 - 64) D. Magnani, O. Barre, S. D. Gerber and M. Solioz. : Characterization of the CopR regulon of *Lactococcus lactis* IL1403, *J.Bacteriol.*, 190(2),536-545 (2008)
 - 65) P. Varmanen, H. Ingmer and F. K. Vogensen. : ctsR of *Lactococcus lactis* encodes a negative regulator of clp gene expression, *Microbiology*, 146 (Pt 6),1447-1455 (2000)
 - 66) P. Varmanen, F. K. Vogensen, K. Hammer, A. Palva and H. Ingmer. : ClpE from *Lactococcus lactis* promotes repression of CtsR-dependent gene expression, *J.Bacteriol.*, 185(17),5117-5124 (2003)
 - 67) M. Fernandez, M. Kleerebezem, O. P. Kuipers, R. J. Siezen and R. van Kranenburg. : Regulation of the metC-cysK operon, involved in sulfur metabolism in *Lactococcus lactis*, *J.Bacteriol.*, 184(1),82-90 (2002)
 - 68) I. Akyol and C. A. Shearman. : Regulation of flpA, flpB and rcfA promoters in *Lactococcus lactis*, *Curr.Microbiol.*, 57(3),200-205 (2008)
 - 69) C. Barriere, M. Veiga-da-Cunha, N. Pons, E. Guedon, S. A. van Hijum, J. Kok, O. P. Kuipers, D. S. Ehrlich and P. Renault. : Fructose utilization in *Lactococcus lactis* as a model for low-GC gram-positive bacteria: its regulator, signal, and DNA-binding site, *J.Bacteriol.*, 187(11),3752-3761 (2005)
 - 70) J. W. Sanders, K. Leenhouts, J. Burghoorn, J. R. Brands, G. Venema and J. Kok. : A chloride-inducible acid resistance mechanism in *Lactococcus lactis* and its regulation, *Mol. Microbiol.*, 27(2),299-310 (1998)
 - 71) J. W. Sanders, G. Venema and J. Kok. : A chloride-inducible gene expression cassette and its use in induced lysis of *Lactococcus lactis*, *Appl.Environ.Microbiol.*, 63(12),4877-4882 (1997)
 - 72) R. Larsen, T. G. Kloosterman, J. Kok and O. P. Kuipers. : GlnR-mediated regulation of nitrogen metabolism in *Lactococcus lactis*, *J.Bacteriol.*, 188(13),4978-4982 (2006)
 - 73) K. Savijoki, H. Ingmer, D. Frees, F. K. Vogensen, A. Palva and P. Varmanen. : Heat and DNA damage induction of the LexA-like regulator HdiR from *Lactococcus lactis* is mediated by RecA and ClpP, *Mol.Microbiol.*, 50(2),609-621 (2003)
 - 74) M. L. Bovee, K. S. Champagne, B. Demeler and C. S. Francklyn. : The quaternary structure of the HisZ-HisG N-1-(5'-phosphoribosyl)-ATP transferase from *Lactococcus lactis*, *Biochemistry*, 41(39),11838-11846 (2002)
 - 75) K. S. Champagne, E. Piscitelli and C. S. Francklyn. : Substrate recognition by the hetero-octameric ATP phosphoribosyltransferase from *Lactococcus lactis*, *Biochemistry*, 45(50),14933-14943 (2006)
 - 76) M. O'Connell-Motherway, D. van Sinderen, F. Morel-Deville, G. F. Fitzgerald, S. D. Ehrlich and P. Morel. : Six putative two-component regulatory systems isolated from *Lactococcus lactis* subsp. *cremoris* MG1363, *Microbiology*, 146 (Pt 4),935-947 (2000)
 - 77) B. Martinez, A. L. Zomer, A. Rodriguez, J. Kok and O. P. Kuipers. : Cell envelope stress induced by the bacteriocin Lcn972 is sensed by the Lactococcal two-component system CesSR, *Mol.Microbiol.*, 64(2),473-486 (2007)
 - 78) H. Agustiandari, J. Lubelski, van den Berg van Saparoea, H. B., O. P. Kuipers and A. J. Driessen. : LmrR is a transcriptional repressor of expression of the multidrug ABC transporter LmrCD in *Lactococcus lactis*, *J.Bacteriol.*, 190(2),759-763 (2008)
 - 79) P. K. Madoori, H. Agustiandari, A. J. Driessen and A. M. Thunnissen. : Structure of the transcriptional regulator LmrR and its mechanism of multidrug recognition, *EMBO J.*, 28(2),156-166 (2009)
 - 80) U. Andersson and P. Radstrom. : Physiological function of the maltose operon regulator, MalR, in *Lactococcus lactis*, *BMC Microbiol.*, 2,28 (2002)
 - 81) B. Cesselin, D. Ali, J. J. Grataudoux, P. Gaudu, P. Duwat, A. Gruss and M. El Karoui. : Inactivation of the *Lactococcus lactis* high-affinity phosphate transporter confers oxygen and thiol resistance and alters metal homeostasis, *Microbiology*, 155(Pt 7),2274-2281 (2009)
 - 82) M. Kilstrup and J. Martinussen. : A transcriptional activator, homologous to the *Bacillus subtilis* PurR repressor, is required for expression of purine biosynthetic genes in *Lactococcus lactis*, *J.Bacteriol.*, 180(15),3907-3916 (1998)
 - 83) J. Martinussen, J. Schallert, B. Andersen and K. Hammer. : The pyrimidine operon pyrRPB-carA from *Lactococcus lactis*, *J.Bacteriol.*, 183(9),2785-2794 (2001)
 - 84) S. M. Madsen, T. Hindre, J. P. Le Pennec, H. Israelsen and A. Dufour. : Two acid-inducible promoters from *Lactococcus lactis* require the cis-acting ACiD-box and the transcription regulator RcfB, *Mol.Microbiol.*, 56(3),735-746 (2005)
 - 85) D. Frees, P. Varmanen and H. Ingmer. : Inactivation of a gene that is highly conserved in Gram-positive bacteria stimulates degradation of non-native proteins and concomitantly increases stress tolerance in *Lactococcus lactis*, *Mol.Microbiol.*, 41(1),93-103 (2001)
 - 86) P. Veiga, C. Bulbarela-Sampieri, S. Furlan, A. Maisons, M. P. Chapot-Chartier, M. Erkelenz, P. Mervelet, P. Noirot, D. Frees, O. P. Kuipers, J. Kok, A. Gruss, G. Buist and S. Kulakauskas. : SpxB regulates O-acetylation-dependent resistance of *Lactococcus lactis* peptidoglycan to hydrolysis, *J.Biol.Chem.*, 282(27),19342-19354 (2007)
 - 87) K. A. Erlandson, J. H. Park, Wissam, Khal El, H. H. Kao, P. Basaran, S. Brydges and C. A. Batt. : Dissolution of xylose metabolism in *Lactococcus lactis*, *Appl.Environ.Microbiol.*, 66(9),3974-3980 (2000)
 - 88) E. Morello, L. G. Bermudez-Humaran, D. Llull, V. Sole, N. Miraglio, P. Langella and I. Poquet. : *Lactococcus lactis*, an efficient cell factory for recombinant protein production and secretion, *J.Mol.Microbiol.Biotechnol.*, 14(1-3),48-58 (2008)